U.PORTO

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

Relatório Final de Estágio
Mestrado Integrado em Medicina Veterinária

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Paulo Joaquim da Silva Santos

Orientador: Professor Doutor Paulo Manuel Rodrigues Vaz Pires
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Abstract

Seasonal variations in both light duration and water temperature are known to be important factors that may affect fish immunity and disease resistance. The present study was thus conceived to evaluate the rainbow trout (*Oncorhynchus mykiss*) immunological status under farming conditions throughout a year. Blood samples were collected at Quinta do Salmão (Pisões, Portugal) from three different groups (diploids, triploids, and female diploids of bigger size) in nine time points. Ten animals from each diploid and triploid group were evaluated at each time point, whereas in the group of fish close to commercial size only five animals were sampled per time. Then, at CIIMAR facilities, blood smears were performed, air dried and the rest of the the plasma collected for humoral parameters quantification. Immune cells were identified and a differential count of neutrophils, monocytes, lymphocytes and thrombocytes was made. Humoral parameters on plasma such as peroxidase, lysozyme and antiprotease activity were measured. Results showed significant differences in all groups with temperature variation, being that fish haematological values showed highest levels of lymphocyte and thrombocyte cells at cooler temperatures and phagocytic cells on warmer waters. Instead humoral parameters showed increased activity on summer months for peroxidase and winter months for lysozyme and antiproteases. Although some of these variations may be caused by fish normal development, genetic or environmental interactions, the evaluation of more immune parameters, including IgM levels in plasma could clarify our hypothesis.

Keywords: rainbow trout, temperature, leukocytes, immune status, peroxidase, lysozyme, antiproteases.

Resumo

As variações sazonais no fotoperiodo e na temperatura da água são conhecidos por serem fatores importantes que podem afetar a imunidade e a resistência a doenças nos peixes. O presente estudo foi assim concebido para avaliar o estado imunológico da truta arco íris (*Oncorhynchus mykiss*) em condições de cultivo ao longo de um ano. As amostras de sangue foram recolhidas na Quinta do Salmão (Pisões, Portugal) de três grupos diferentes (diploides, triploides e diploides femininos de tamanho maior) em nove períodos temporais. Foram avaliados dez animais de cada grupo diploide e triploide em cada amostragem, enquanto que no grupo de peixes perto do tamanho comercial apenas cinco animais foram amostrados por tempo. Seguidamente, nas instalações de CIIMAR, foram realizados esfregaços de sangue, secos ao ar e o resto do plasma recolhido para quantificação de parâmetros humorais. Os leucócitos foram identificados e uma contagem diferencial de neutrófilos, monócitos, linfócitos e trombócitos. Foram medidos parâmetros humorais no plasma, tais como atividade da peroxidase, lisozima e antiprotease. Os resultados mostraram diferenças significativas em todos os grupos com a variação da temperatura, sendo que os valores hematológicos de peixes apresentaram níveis mais altos de linfócitos e trombócitos a temperaturas mais baixas e células fagocíticas em águas mais quentes. Em vez disso, os parâmetros humorais mostraram aumento da atividade nos meses de verão para a peroxidase e nos meses de inverno para a lisozima e as antiproteases. Embora algumas dessas variações possam ser causadas pelo desenvolvimento normal dos peixes ou por interações genéticas ou ambientais, a avaliação de mais parâmetros imunes, incluindo níveis de IgM no plasma, poderiam esclarecer a nossa hipótese.

Palavras-chave: truta-arco-íris, temperatura, leucócitos, sistema imune, peroxidase, lisozima, antiproteases.

This thesis also includes one scientific poster with the title “Seasonal Blood Cell Dynamics in Farmed Rainbow Trout: A Comparative Study Between Diploid and Triploid Fish”, presented at the 2nd AquaImprove, Aquaculture Research Workshop, that took place on 17th March 2017 at CIIMAR (Interdisciplinary Centre of Marine and Environmental Research) facilities: Terminal de Cruzeiros do Porto de Leixões, Avenida General Norton de Matos, S/N, 4450-208 Matosinhos, Portugal (Appendix).

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1. Introduction

1.1 Evolution of World and European Aquaculture

World's fish market has been rising over the last 50 years, following population growth and food consumption, being that fish constitutes an important source of nutrients and proteins for several people all over the globe (FAO 2012). In 2010, fish consumption represented about 17 percent of animal protein ingestion and 6.5 percent of total protein ingested (FAO 2014). Already in 2014, World per capita apparent fish consumption reached a new record, fixed in 20.2 kg compared to 9.9 kg in 1960s, and is estimated that till 2025 it hits 21.8 kg, corresponding to a growth of 7.8 percent. At the same time, the supply of fish has accompanied this growth from the 1960s to the present day, mainly due to the contribution of aquaculture. While almost 40 million tonnes of fish were produced in 1960, almost exclusively from capture, in 2014, about 168 million tonnes were produced, of which 93.4 million tonnes came from capture and 73.8 million tonnes are the result of aquaculture production, accounting for about 44 percent of world fish production (Figure 1).

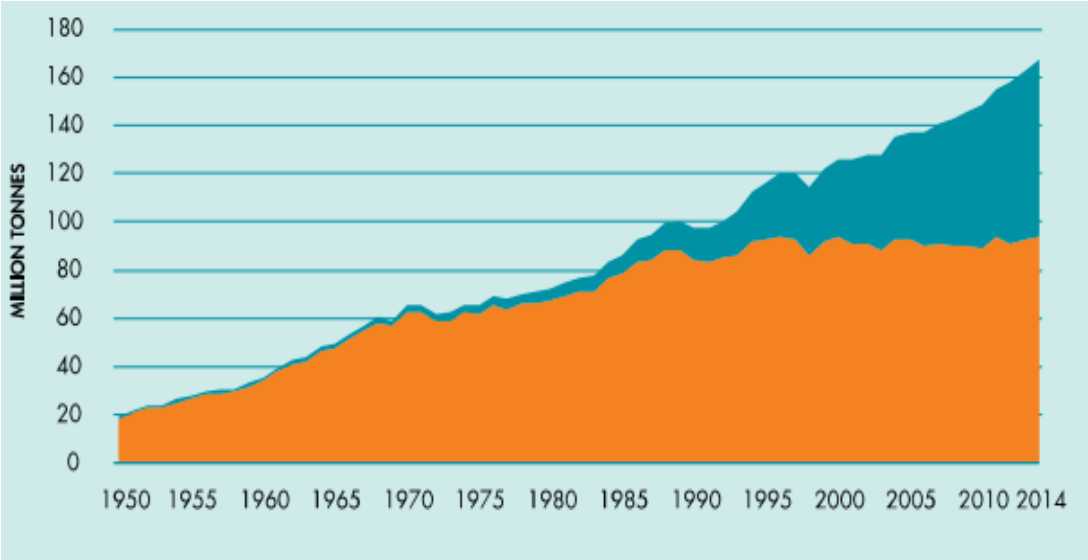

Figure 1- World seafood production by capture ( ) and aquaculture ( ): 1950-2014. Source: FAO 2016

The culture of aquatic organisms is organized into finfish, molluscs, crustaceans, amphibians and other reptiles and aquatic plants. This is a very important way of production for the sustainability of the human race since it allows us to increase the quantity and quality of sea products at our disposal with relative ease, contrary to fishing that, although remained stable in

the last years, is too much dependent on non-controllable factors such as food supply, predation, water pollution and global warming (United Nations Development Programme 2012).

The main producers come from Asia, where about 55 percent of the aquatic animals consumed are produced in aquaculture, being China particularly important because is one of the pioneers in fish cultivation and has produced about 58 795 thousand tons in 2014. In Europe, although it only represents 18 percent of the supply, aquaculture production has been increasing, doubling the percentage of cultivated fish in 1995. Thus, according to the Food and Agriculture Organization, 2014 Europe's production reached 2,930 million tonnes, with the Southern Europe countries, where Portugal is included, contributed with 595 thousand tonnes (FAO 2016).

More specifically, in Portugal and the same year, according to Instituto Nacional de Estatistica, about 10,791 tonnes of marine animals were produced in aquaculture. In our territory fish are the main group cultivated, of which we can emphasize *Scophtalmus maximus* (turbot), *Sparus aurata* (gilthead seabream), *Dicentrarchus labrax* (European seabass) and *Oncorhynchus mykiss* (rainbow trout). Regarding the exploitation regime, we can affirm that in fresh water Portugal produces exclusively in an intensive way, while in brackish and marine waters 47.8 percent of the production was obtained extensively, 39.2 percent intensively and the remaining percentage by the semi-intensive regime (INE 2016).

1.2 Characterization of the species

Oncorhynchus mykiss (Walbaum, 1792) is a fusiform fish of the Actinopterygii class, Teleostei infra-class and Salmonidae family. It is a freshwater fish with a brownish or yellowish body, with black spots over the length of the body and a pinkish stripe that extends from the gills to the caudal fin, which is a specific characteristic of this species (Figure 2). Its origin is the Pacific coast of the United States, although at present it is widely distributed, both because of its high resistance and environmental adaptability and of its crescent demand. Its introduction in Europe took place at the end of the 19th century and is currently being created all over Europe, being mainly exported by countries such as Denmark, Poland and Sweden. In our country, the creation of *O. mykiss* dates from the beginning of the 60s, with the first unit to be installed on the Coura River (A Pesca e a Aquicultura na Europa 2012).

Figure 2- *Oncorhynchus mykiss* (Walbaum, 1792)

Its production in Portugal occurs exclusively under intensive mode in fresh water and, in 2014, 787 tons of *O. mykiss* were produced (INE 2016). *O. mykiss* growth and maturation are influenced by water temperature and feed, which must be high in protein, and maturity is reached between 3 and 4 years of age. Regarding to breeding, this process should ideally be performed with water temperatures below 12º C. After hatching, the larval culture of these animals is made in circular fiberglass or concrete tanks, under conditions of regular flow and balanced distribution of animals. At the moment little fish reach a weight of 50 grams and a size of 8 to 10 centimetres, they are transferred to growing tanks, in floating cages placed in ponds or rectangular concrete fish farms, over a river. Although these cages do not require human intervention for water renewal, in fish farms it represents a task that can be performed by two techniques: through an open system, passing the river water through a channel, or with a closed (recirculation) system, which circulates the water in the tanks and recycles it. After reaching this stage, commercial trout are harvested with a net or pumped by a system that takes them ashore to then be packaged and transported. Besides few studies have been performed to evaluate the presence of pathological

agents on portuguese trout farms, epidemic cases occurred mainly by bacterial Aeromonas salmonicida (Saraiva *et al.* 1989), responsible for furunculosis, and *Yersinia ruckeri* (Sousa 1996).

1.3 Difficulties of aquaculture production

Although our coast has a supposed great potential for aquaculture practice, the truth is that the coasts of the west and north of our territory suffer a great influence of the Atlantic Ocean by its currents and waves, affecting the ambient and water temperature, especially in the winter (Ramalho & Dinis 2010). A recent method that is being used in fish farms is the offshore, where fish cages are moved and submerged in deeper water in order to increase fish welfare, decrease pathologies and dilute wastes from fish production (Naylor & Burke 2005). Although this method assumes an ecological and spacial concern which is of relevant importance for aquaculture producers to obtain their farming licenses, the offshore technology represents a challenge to fish farmers since these structures are very susceptible to storms and wind (Stickney *et al*. 2006), with some reported cases of cage damages, resulting in considerable economical losses around the world.

Investment in fish feed is also very important, representing a large share of aquaculture expenditure. The food supplied must be adapted to the species, nutritional needs and stage of life, whereby producers, by relying on nutritional management companies, benefit from better information on handling, packaging and nutritional value, as well as tools that allow them to evaluate production indexes such as feed conversion and growth rates to quickly detect food deficiencies and corrected them (Ramalho & Dinis 2010; Hasan & New 2013).

Another obstacle that influences marine products is the poor modernization, either by the protection policies of the coastal zones or by the costs inherent in it, which are incompatible with our small and familiar farms (MADRP-DGPA 2007). Recirculating aquaculture systems (RAS) are innovative and extremely efficient, using tanks that permit intensive fish production with limited water exchange, due to biofiltration and other environmental correction resources that keep water clean resulting in an healthier environment (Timmons & Ebeling 2013). Beyond the above profits, RAS brings to producers higher flexibility to choose a place to install a fish farm, higher stock density and production by area unit, drastic reduction of the effluent volume, water parameters control such as pH, oxygenation and temperature, as well as fast access to feed and cultivated animals that lead to enhanced performance. Besides all these advantages, RAS is still not being widely explored in Portugal because of the high upfront investment in material and infrastructures, as well as the need of experient and trained staff to monitor and operate the systems (Rawlinson & Forster 2000).

1.4 Haematology

Blood studies in fish are relatively recent being the first reports of observation of erythrocytes in 1845 by Gulliver, and of leukocytes in 1905 by Drzewina (Oria 1932; Ellis 1976; Ellis 1977). The execution of blood smears is of great importance since it allows us to assess the immunological status of the fish in a very quick and representative way, in which only one drop of blood is enough for a preliminary evaluation. However, its realization requires practice since the distinction between the various lymphoid cells represents a complex task. To a better comprehension of this subject, a brief summary of the most representative lymphoid cells on *O. mykiss*'s blood will be presented (Figure 3).

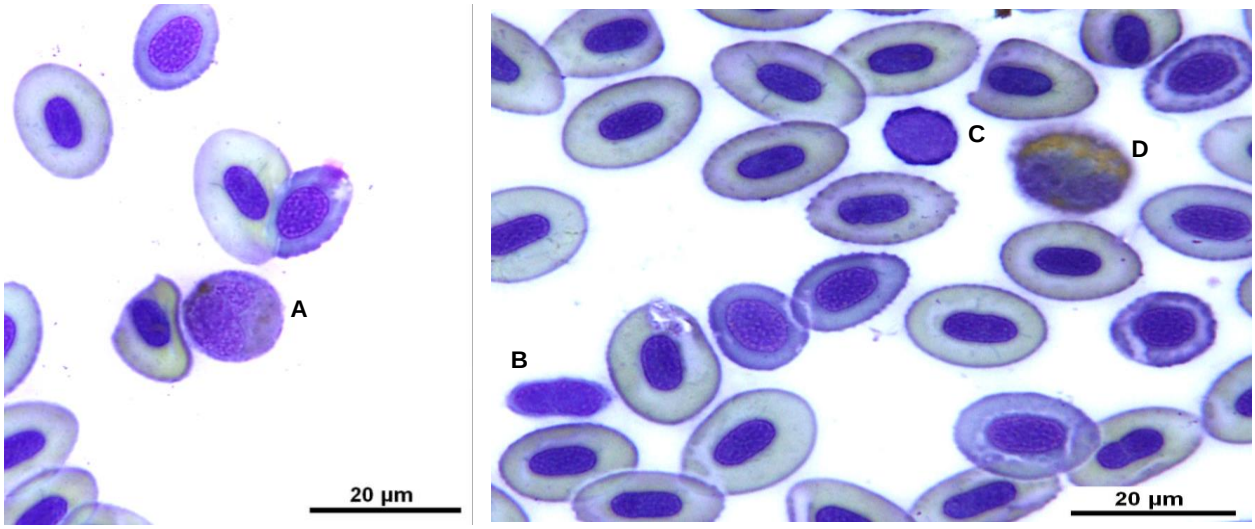

Figure 3 - Blood *O. mykiss* smears stained with Wright's stain. Neutrophils were labelled using the Antonow's technique presenting abundant peroxidase granules. Letters represent: (A) monocyte; (B) thrombocyte; (C) lymphocyte; (D) neutrophil.

Lymphocytes are the most representative lymphoid cells in *O. mykiss* blood. They are predominantly rounded cells, of variated size, with basophilic cytoplasm and without granulations. Its nucleus presents dense chromatin and is frequently observed an high nucleus/cytoplasm rate. Although they may resemble thrombocytes, lymphocytes show cytoplasmic projections, which facilitates their differentiation (Tavares-Dias & Moraes 2004).

The neutrophils are predominantly rounded, whose cytoplasm has very fine greyish granulations and the nucleus has the form of a rod, peripheral, with the nuclear chromatin not very compact and without a visible nucleolus. These cells have high migratory activity, like that

occurring in mammals, and a strong non-specific cytotoxic activity (Griffin 1984; Sasaki *et al*., 2002). In addition to these characteristics, the presence of peroxidase, a lysosomal enzyme, promotes the oxidation of compounds by hydrogen peroxide in the phagocytosis process (Oliveira *et al.* 1997).

The monocytes are recognized as cells with few pseudopods, large numbers of mitochondria and vacuoles, and some endoplasmic reticulum and Golgi complex. Its nucleus occupies a third to a quarter of the cell and using optical microscope it is possible to observe them quite dense, surrounded by a basophilic cytoplasm, distinctive from other cellular groups (Dogget & Harris 1989). There is some confusion about the denomination of these cells due to their phagocytic action, with some authors considering them macrophages. However, the monocyte/macrophage designation is simple since monocytes act as circulating cells while macrophages migrate to tissues and other places like the peritoneal cavity and natatory bladder (Lorenzi 1999; Lamas *et al.* 1994).

The last lymphoid cells are the thrombocytes, which are observed under the optical microscope as elliptic cells, with fusiform and colorated nucleus. These cells have the same functions of platelets in mammals and can be found in birds, reptiles, amphibians and fish, with the function of haemostasis and homeostasis (Roberts 1981). Thrombocytes are also in discussion about their inclusion as lymphoid cells, being that past researchers have demonstrated the phagocytic function of thrombocytes, suggesting these cells to make part of the defence cell group (Hill & Rowley 1988). There is also evidence of presence of pattern recognition receptors (PRR) on thrombocytes membrane that act by recognition of pathogen-associated molecular pattern (PAMP) and damage-associated molecular pattern (DAMP) present on pathogens and host cells respectively.

1.5 Immune system and inflammation

Besides fish being one of the primitive vertebrates, they possess phagocytic mechanisms that work side by side with humoral and cell-mediated immunity (Roberts 2012). After infection, immune system can act by two different ways, according to response speed and specifity. The first response is from the innate system, that is fastest entering in action but low specific. Although that, fish innate system is able to discriminate self and non-self organisms, and his role is to protect the host by inhibiting pathogen's multiplication (Jaqueline Parkin 2001). Later comes the adaptive immunity, a pathogen specific response caused by antigen presentation from myeloid cells to lymphocytes, with antibody and memory cell production for that kind of infection, on a process that is influenced by temperature (Ellis 1999). Lymphocytes are cells of great relevance for adaptive immunity, once different cell type lead to antigen recognition, specificity and memory. These cells can be divided into B-cells responsible for antibody production (on fish the most produced antibodies on systemic circulation are IgM, while IgT is widely present on mucosal surface),and T-cells that mediate cell-mediated immunity. T-cells can be subdivided according to their direct action on killing infected and abnormal cells (cytotoxic T-cells), or to them modulation activity to other cells through cytokine production (helper T-cells) (Gudding *et al.* 2014).

Phagocytosis is the primordial cell defence mechanism activated after injury or infection and is modulated by phagocytes, that can be divided into professional phagocytes (such as neutrophils, monocytes and macrophages), with receptors on their surface able to detect harmful objects, and non-professional phagocytes (Corbel 1975). In aquaculture, there is a particular interest at developing disease's resistance by increasing the phagocytic activity of defence cells. The phagocytic process occurs due to recognition and attachment to a foreign particle, with posterior engulfment and digestion. (Roberts 2012). Macrophages are cells that enter in action after different types of activation, being the most common designation M1 or M2, according to lymphocyte T helper 1 (Th1) or T helper 2 (Th2) cytokine induction (Mantovani 2002). However, recent studies have enlarged the macrophages phenotype to four, according to different environmental signals (Forlenza *et al.*, 2011). Firstly, we have innate activation of macrophages, that occurs after an isolated microbial stimulus which is recognized by macrophage's Toll-like Receptors (TLR), Cluster of differentiation (CD)14 or other PRR's. Similarly to the above, we have classical activation, that requires the same stimulus of innate activation plus the presence of the cytokine Interferon ɣ (IFN-ɣ) (Dalton *et al.* 1993). The third and fourth phenotypes, alternative activated macrophages and regulatory macrophages, are associated with bacterial interaction with Interleukin-4/Interleukin-13 and Interleukin-10 respectively.

Other host defence mechanisms that try to contain the propagation or eliminate the cause of host-tissue damage are called by some authors as nonspecific mediators of immunity. They

act as physical barriers to invading organisms before cell activation occurs of which we can highlight skin and mucus. The mucosal surfaces of fishes (gill, skin, and gastrointestinal tract) form a thin physical barrier between the external environment and the internal milieu, and they are important sites of microbial exposure. Cutaneous mucus is considered the first line of defence against infection through skin epidermis and it acts as a natural, physical, biochemical, dynamic, and semipermeable barrier that enables the exchange of nutrients, water, gases, odorants, hormones, and gametes (Esteban 2012). Furthermore, mucus in fish has the ability to trap and immobilize pathogens before epitelial surface contact, due to its constant secretion and substitution (Mayer 2003). In fish mucus, immune system components have been identified such as enzymes, proteases, antimicrobial peptides, lectins, proteins and immunoglobulins, that can provide a protective role for aquatic organisms.

After infection or tissue lesion, an inflammatory process begins in order to repair the damage and stablish homeostasis (Kiron 2012). Inflammation mechanism is multi-factorial and still few studied in fish (Ellis 2001), but is yet known that vascular phenomena occurs, initiating a fast and local increased blood flow, resulting in hyperaemia. After this, local inflammatory mediators are produced, promoting a process called chemotaxis, calling neutrophils and monocytes to further phagocytosis (Kindt *et al.* 2007). Phagocytic cells are also capable of inhibit the colonization, survival and proliferation of microorganisms with a set of antimicrobial agents such as lysozyme, complement factors (Barton 2008), antiproteases (Secombs 1996), and bactericidal reactive oxygen species (Ellis 2001).

Lysozyme is a phagocytic cell's enzyme which hydrolyses N-acetylmuramic acid and N-acetylglucosamine which are constituents of the peptidoglycan layer of bacterial cell walls. The complement system can be activated by antigen-antibody reactions or by the so called alternative route, via binding to microbial cell wall polysaccharides, which results in opsonization and/or lysis of foreign cells (Bayne & Gerwick 2001). Antiproteases are substances that have yet been found in *O. mykiss* serum (Ellis *et al.* 1981) and their role is to inhibit the proteases released from bacteria that help these pathogens to use host proteins as substrate for their maintenance. The main protease inhibitors are α1-anti-protease, α2-anti-plasmin and α2-Macroglobulin (α2M) (Ellis 2001). Neutrophils contain myeloperoxidase (MPO) in their cytoplasmic granules (Afonso *et al*. 1997). MPO in the presence of halide ions and H_2O_2 can kill bacteria by halogenation of the bacterial cell walls as well as production of bactericidal hypohalite ions (Klebanoff & Clark 1978).

1.6 Water biotic and abiotic factors in fish haematology and immunity

1.6.1 Fish growth and maturation and genetical improvements

All physiological processes on animal organism occurs due to energy trades, coming from their intermediary metabolism with further channelling of the amount required for each biological task, according to each life phase (Myrick 2011). Fish growth and maturation are two of the most exigent phases of young fish which demand a large quantity of energy and nutrients. Besides there are not many studies that compare fish growth and haematological/leukocytary parameters, DeWilde and Houston have demonstrated that erythrocyte number decreases with *O. mykiss* fish development and weight gain. Likewise this fact, is also demonstrated that younger fish possess higher erythropoietic activity than older and bigger fish (Zhiteneva & Gorislavskaya 1986), which make believe that different size and age aquatic animals produce different haematological values. Although relevant, this parameter shouldn't be considered isolatedly, once fish growth is affected by nutritional and ecophysiological conditions such as ambiental and water temperature, photoperiod and water physical and chemical parameters (Chaudhuri *et al.* 1986).

Posterior studies have been performed on teleosts to verify the influence of fish sex and gonadal maturation on haematological/leukogram values. One of these studies have compared female and male *O. mykiss*, evaluating their leukogram, resulting in no significant differences between different sex groups (Ranzani-Paiva *et al.* 1998a). On the other hand, the same author observed significant differences between erythrogram/leukogram results and gonadal maturation, whose results presented increased erythrocyte, neutrophil and monocyte number, with decrease of lymphocyte number (Ranzani-Paiva *et al.* 1998b), suggesting that gonadal maturation might be one of the physiological factors responsible for blood cell variations.

Increased aquaculture's products demand has opened way for genetical improvements on fish production, with *O. mykiss* triploid or 3N fish started being developed in middle 80´s, by subjecting eggs to thermal or pressure shocks shortly after fertilization (Lincoln & Scott 1983). This technology consists in selected genetic organisms that have high percentage of sterile females. This is one of the genetical developments widely used in aquaculture, since it allows transference of energy that would be used in reproduction to growth. Investigations into non-specific, humoral immune parameters comparing diploid and triploid *Salmo salar* groups produced interesting findings, with antiprotease activity results presenting lower concentrations in triploids (Langston *et al.* 1997).

1.6.2 Stress and parasitism

Fish immune system might be affected by regular farming stressing procedures that are identified at four levels: handling, confinement, stocking and transport (Wendelaar Bonga 1997; Costas *et al.* 2012). These procedures develop physiological deregulation, followed by changes on metabolism and cell processes, especially on leukocyte synthesis, with innate defence mechanisms commitment and increasing the predisposal to pathological situations (Ellis 2001, Tort 2011). One of the most used indicator to evaluate teleost fish on stressing processes is cortisol (Mommsen *et al.* 1999), with increased plasma concentration in affected animals. High cortisol plasma levels may lead to decreased lymphocyte number, supressed phagocytic activities in head kidney and blood, and increased susceptibility to infection (Ortuño *et al.* 2001). Studies made to evaluate cortisol influence on disease predisposal were performed, resulting in increased susceptibility to furunculosis infection by *A. salmonicida* after exogen cortisol administration (Pickering 1989*;* Pickering *et al.* 1992). Stress factors such as transport, anoxia, social conflict, handling, injection and crowding have also been studied resulting in decreased number of lymphocytes and thrombocytes and increased number of circulating neutrophils in several species. (Pickering and Pottinger 1987; Pulsford *et al.* 1994; Espelid *et al.* 1996).

Fish exposition to pathogenic agents may generate physiological response similar to stressant situations, resulting in host immune suppression (Ruane *et al.* 2000). Pathogens can live on host without cause them any immunological change. This coexistence benefits both organisms but when a change on the ambient-pathogen-host system occurs, fish defences are at risk, which can result in a pathological process (Tavares-Dias *et al.* 1999), that in extreme cases ends with death. Infectious agents have different incidence along the year, with bacteria and parasite infections being associated with spring die-offs and viruses commonly affecting fishes in autumn. A study developed on *O. mykiss* parasited with protozoan *Ichthyophonus hoferi* resulted in a lower level of erythrocyte number and haemoglobin concentration (Rand & Cone 1990). The same study revealed an immunosuppression after parasite infection resulting in leukopenia. Another study with *Vibrio anguillarum*, have produced low leukocyte levels too, but was more specific about leukocitary changes, showing decreased lymphocyte levels and increased neutrophil and monocyte levels (Lamas *et al.* 1994). *O. mykiss* was also infected with *Renibacterium salmoninarum* revealing some differences comparing to the results above, particularly at thrombocyte levels that were increased in infected animals (Bruno & Munro 1986).

1.6.3 Water dissolved oxygen and pH

Oxygen concentration is a water parameter of high relevance for fish population, with O_2 playing an essential role on metabolic processes of nutrient assimilation (Castagnoli 2000). Dissolved oxygen values vary on inverse sense of temperature, and oxygen solubility clearly affects inorganic phosphate intra-erythrocitary such as adenosine triphosphate (ATP) and guanosine triphosphate (GTP) (Val 2000). Although some fish can tolerate low oxygen concentration, it clearly affects their health and performance (Noga & Francis-Floyd 1991), being one of the most stressing ambiental factors for fish that requires adaptative adjustments at all biological organization levels (Baldisseroto 2002). Researchers have submitted *O. mykiss* to low concentrations of oxygen which resulted in increased gill ventilation to regulate oxygen distribution and avoid blood pH decrease. These authors have also concluded that low oxygen concentration affects hematological parameters, increasing haematocrit and haemoglobin concentration without erythrocyte number alteration (Holeton & Randall 1967). On an opposite situation, hyperoxia can lead to decreased ventilation frequency, resulting in CO_2 accumulation in blood, respiratory acidosis (Dejours 1977) and imbalance in gill ion concentration (Brauner *et al.* 1999). Salmon fish farms usually supplement oxygen to supersaturate the water with O_2, since they believe it can improve fish growth and increase pathogen resistance (Caldwell and Hinshaw 1994). Although immune response is still not correlated with water oxygen saturation, some studies have demonstrated that moderate hyperoxia can in fact improve fish growth (Hosfeld *et al.* 2008).

Water pollution may change biochemical quality of aquatic animals, causing several problems on fish performance due to water acidification. pH variations can decrease fish activity and swimming skills and alkaline pH levels are required for farming fish performance, with values varying from 7.0 to 8.5 (Baldisseroto 2002). Acid pH is known to affect haemoglobin oxygenation capacity, resulting in low oxygen arterial levels that demand circulating erythrocytes to compensate oxygen transport (Houston & Gingra-Bedard 1994). In addition to other physiological changes, hematologic and leukocyte alterations have also been reported with pH variations. Diverse fish species studies have concluded that low pH levels presented increased haematocrit, total leukocytes and thrombocyte, neutrophil and monocyte percentage (Giles *et al.* 1984; Dheer *et al.* 1987; Rambhaskar & Srinivasa-Rao 1989).

1.6.4 Water Temperature

Fluctuations in water temperature are a seasonal phenomenon which influences humoral, cell mediated and non-specific (physical barriers) defence mechanisms in fish. Seasonal variation affects mainly the temperated zone, due to photoperiod and temperature changes that modulate immunologic mechanisms of fish (Zapata *et al.* 1992). Haematological changes in *O. mykiss* during the year resulted in reduction of erythrocyte number, haematocrit and haemoglobin concentration on the transition autumn/winter (Lane 1979), while summer values showed increased number of erythrocytes and mean corpuscular volume, with opposite decreased haemoglobin concentration and mean corpuscular haemoglobin concentration (Rehulka 1997). Leukogram changes are also observed resulting in higher leukocyte average number during spring, while lymphocyte average number decreased and neutrophil average number increased during summer months. (Houston *et al.* 1996). There are also evidences indicating that fish possess low immunological activity on winter, similarly to what happen with amphibian and reptiles (Zapata *et al.* 1992).

It is globally recognized that environmental factors and water temperature affect teleost growth, reproduction, survival and metabolism. A study focused on the performance of female *O. mykiss*, comparing diploid and triploid animals at chronic high temperatures, have showed that triploid animals were more susceptible to water temperature variations in terms of growth and survival (Ojolick *et al.* 1995). Increased water temperature leads to more biological and metabolic activity of tropical fish, with consequent increase in the respiratory and cardiac frequencies due to bigger oxygen demand (Baldisserotto 2002). Haematocrit, haemoglobin concentration and erythrocyte number show tendency to increase with water temperature elevation (Martinez *et al.* 1994). Water temperature variations produce diverse leukogram discrepancies in different teleost species. In 2002, studies have concluded that temperatures between 15 and 17 ºC produces bigger monocyte, granulocyte and B lymphocyte activation on *O. mykiss* after inoculation with *A. salmonicida* than with temperatures between 10 and 12 ºC. However, the development of a specific antibody response against infection seemed to be more effective at lower temperatures (Kollner & Kotterba 2002). Also on study specie, phagocytosis increases with temperature elevation, compensating the lymphocytopenia (Houston *et al.* 1996). Between phagocytic cells, neutrophils seemed to be the most resistant defence cells to immunosuppression with low temperature (Ainsworth *et al.* 1991). Another study developed on *Salmo trutta lacrustis* (brown trout) kept at 15 ºC for 77 days have produced lowest leukocyte and thrombocyte values, with no significative alteration on number of lymphocytes or neutrophils (Rahkonem & Pasternack 1998).

1.7 Scope of study

Although aquaculture is a valid alternative for food sustainability, it represents a big investment and is still somehow insecure due to problems of population density, fish immune system and pathologies associated with some mortality and consequently monetary losses. In view of these problems and the scarcity of studies in natural conditions of this species, the objective of this work is to verify the influence of water temperature on the immunological status of *O. mykiss* under culture conditions, through monthly samplings along all seasons of the year.

The chosen aquaculture was Quinta do Salmão, installed on the Portuguese district of Vila Real, in the municipality of Montealegre, at Rio Rabagão's dam, where *O. mykiss* are cultivated on cages on an intensive way (Figure 4). These cages could be circular or rectangular and rainbow trouts were separated in three different groups: one group of diploid small fishes (average fish weight of 6.5 grams at the beginning of the samplings), constituted by male and female fish that are descendants from reproductors of this aquaculture; one group of imported triploid female small fishes (average fish weight of 7.5 grams at first sampling); and one group of medium sized female animals (average fish weight of 300 grams at first time sampling) that were used on this aquaculture as reproductors. As this fish farm is placed on a valley and cages are placed on the river and not covered, fish are clearly susceptible by water temperature fluctuations which might affect animal's immune defences.

The immune status of two *O. mykiss* populations genetically different was evaluated, throughout a year. Moreover, female diploid fish of bigger size were also evaluated at the same time to assess eventual size/age effects. Fish cellularity and humoral parameters as lysozyme, peroxidase and antiprotease activity were measured to identify periods of immunosuppression at each group, in order to later implementation of prophylactic measures that may improve the immune resistance of fish in critical periods.

Figures 4- Images of Quinta do Salmão aquaculture, at Rio Rabagão's dam, Pisões.

2. Materials and Methods

2.1 Sampling

The sampling of animals was carried out monthly between June 2016 and March 2017 with water temperature recording as shown (table I). Each time sampling, blood was collected from 10 small diploid fish, 10 small triploid fish and 5 medium/large animals. The animals were anesthetized with 2-phenolyethanol (Merck) and mucus was gently collected in a 15 ml Falcon tube and stored at -20 ºC until assayed. Afterwards, fish were weighed and measured. The blood was then collected through the caudal vein through a vacuum system with heparin, stored in heparinized tubes and kept on ice until arrival at the CIIMAR facilities.

Table I. Time sampling and water temperature registration

Time Sampling	Water Temperature
Time 1 – 21/06/16	18.0 ºC
Time 2 – 08/07/16	22.0 ºC
Time 3 – 07/09/16	22.0 ºC
Time 4 – 02/11/16	15.4 ºC
Time 5 – 25/11/16	11.0 ºC
Time 6 – 19/12/16	09.2 ºC
Time 7 – 30/01/17	06.6 ºC
Time 8 – 24/02/17	08.0 ºC
Time 9 – 24/03/17	08.0 ºC

2.2 Haematological analysis

Upon arrival in the laboratory, the smears from heparinized blood were run through a uniform blood droplet and air dried, the rest of the blood being centrifuged at 10,000 × *g* for 10 min at 4 ºC and plasma was collected and frozen at -80 °C for humoral parameter quantification. Note that for the first time sampling no blood smears were performed. After air drying, the slides were fixed with a solution of formaldehyde-ethanol (90% absolute ethanol to 10% of 37% formaldehyde) for one minute (Kaplow 1965). Neutrophils were identified by detection of peroxidase activity, following a protocol described by Afonso et al (1998). Afterwards, slides were stained with the Wright's stain (Haemacolor, Merck) and observed under oil immersion (1000X). Immune cells were identified and a differential count of neutrophils, monocytes, lymphocytes and thrombocytes was made in a total of 200 cells/smear.

2.3 Humoral parameters analytical procedures

1) Lysozyme: Lysozyme activity was measured using a turbidimetric assay as described by Costas et al. (2011). First, a solution of *Micrococcus lysodeikticus* (0.5 mg ml^{-1}, 0.005 M sodium phosphate buffer, pH 6.2) was prepared. After this 15 µl of plasma and 250 µl of the above suspension were added to a microplate to achieve a final volume of 265 µl. The reaction was carried out at 25º C and the absorbance (450 nm) was measured after 0.5 and 4.5 minutes in a Synergy HT microplate reader, Biotek (Figure 5). Lyophilized hen egg white lysozyme (Sigma) was serially diluted in sodium phosphate buffer (0.05 M, pH 6.2) and used to develop a standard curve. The amount of lysozyme in the sample was calculated using the formula of the standard curve. All analysis was conducted in duplicates.

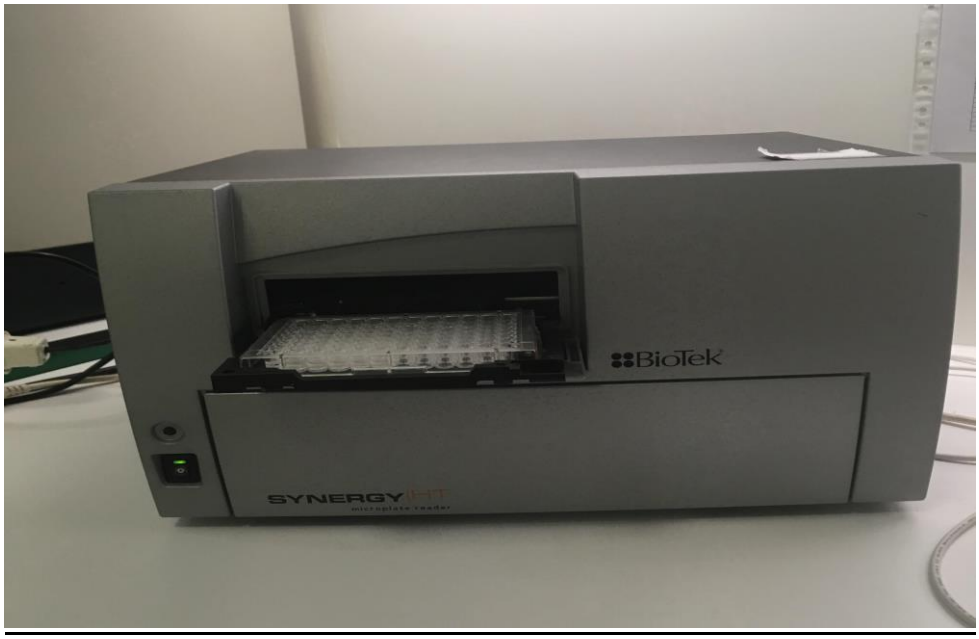

Figure 5- Synergy HT microplate reader, Biotek, before reading lysozyme activity (450 nm).

2) Peroxidase activity: Total peroxidase activity in plasma was measured following the procedure described by Quade and Roth (1997). Firstly, we diluted samples in HBSS on a 1:10 dilution. After this a 15 µl of the dilution above were diluted in 250 µl of HBSS without Ca^{2+} and Mg^{2+} in flat bottomed 96-well plates. Then, 50 µl of 20 mM 3,3',5,5'-tetramethybenzidine hydrochloride (TMB; Sigma) and 50 µl of 5 mM hydrogen peroxide were added (Figure 6). The colour change reaction was stopped after 2 minutes by adding 50 µl of 2M sulphuric acid (Figure 7) and the optical density was read at 450 nm in a Synergy HT microplate reader, Biotek. The wells without plasma were used as blanks. The peroxidase activity (units ml^{-1} plasma) was determined defining one unit of peroxidase as that which produces an absorbance change of 1 Optical Density (OD).

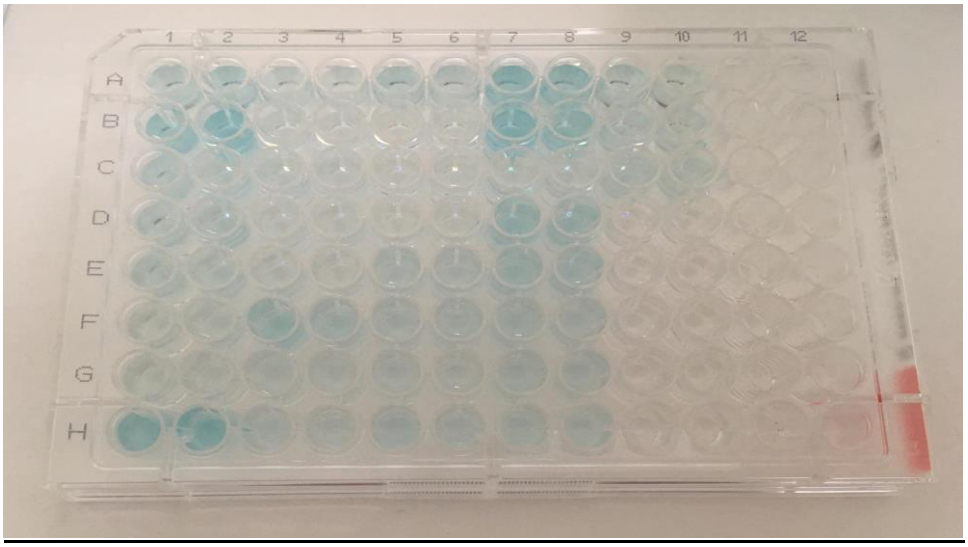

Figure 6- Microplate after adding hydrogen peroxide.

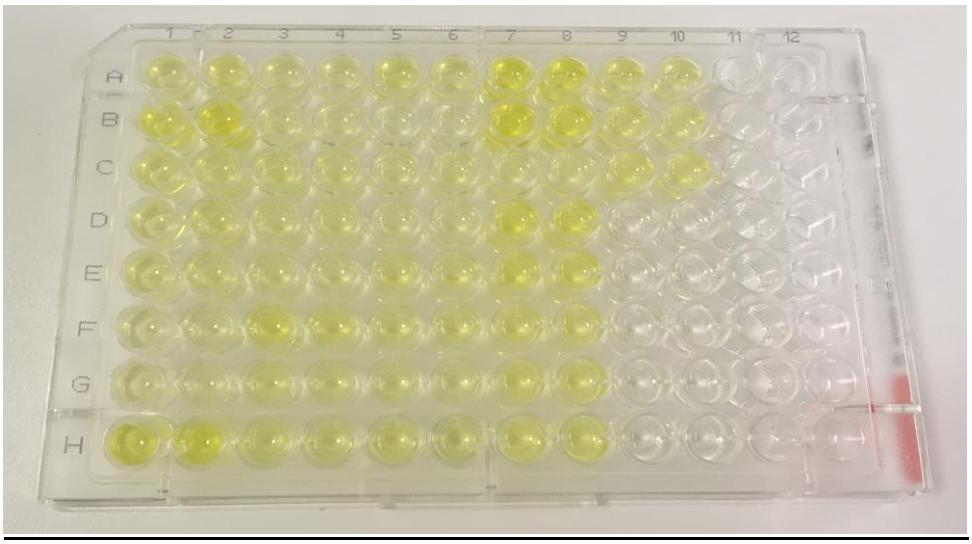

Figure 7- Microplate after adding sulphuric acid.

3) Antiprotease Activity: The method described by Ellis (1990) was modified and adapted for 96-well microplates (Machado *et al.* 2015). At first, 10 µl of plasma were incubated with the same volume of trypsin solution (5 mg ml^{-1} in $NaHCO_3$ 5 mg ml^{-1}, pH 8.3) for 10 minutes at 22 ºC in polystyrene microtubes. To the incubation mixture, 100 µl of phosphate buffer (NaH_2PO_4, 13.9 mg ml^{-1}, pH 7.0) and 125 µl of azocasein (20 mg ml^{-1} in $NaHCO_3$, 5 mg ml^{-1}, pH 8.3) were added and incubated for 1 h at 22 ºC. After this, 250 µl of trichloroacetic acid were added to the microtubes and incubated for 30 min at 22 ºC. Finally, the mixture was centrifuged at 10,000 × *g* for 5 min at room temperature. The blank was made using phosphate buffer saline instead of plasma and trypsin, and the reference sample was obtained using phosphate buffered saline instead of plasma (Figure 8). To calculate the percentage of trypsin activity we used the following formules:

% non-inhibited trypsin = (Sample absorbance × 100) / Reference sample

% inhibited trypsin = 100 - % non-inhibited trypsin

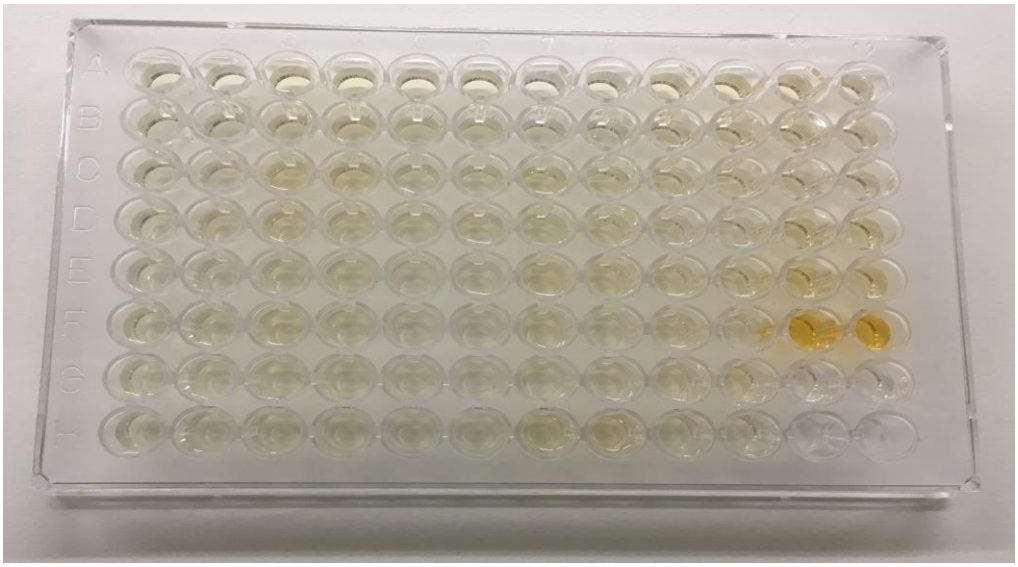

Figure 8- Antiprotease microplate before reading.

2.4 Statistical analysis

The groups of animals were divided by size/genetic group and, for each time sampling and parameter, mean and standard deviation were calculated. Data were analysed for normality and homogeneity of variance and Log or Arc Sen transformed before statistical treatment when needed. Data were analysed by one-way ANOVA (Tukey post hoc test) when normality and homogeneity was observed. Alternatively, Nonparametric Kruskal-Wallis test was performed to find significative changes of sampling parameters between time sampling groups. The performance of statistical analyses occurred under STATISTICA 13 program for WINDOWS. The level of significance used was $p \leq 0.05$ for all statistical tests.

3. Results

Haematological studies have shown that, on diploid fish, water temperature variation have not affected neutrophils, lymphocytes and thrombocytes, just producing little changes on monocyte count, with a significant increase between 22.0 ºC 8.0 ºC (Table II).

Table II- Relative proportion of diploid *O. mykiss* peripheral blood leukocytes (thrombocytes, lymphocytes, monocytes and neutrophils) for each time sampling

	22.0 ºC	**22.0 ºC**	**15.4 ºC**	**11.0 ºC**	**9.2 ºC**	**6.6 ªC**	**8.0 ºC**	**8.0 ºC**
Neutrophils (% WBC)	3.65 ± 2.07	6.10 ± 5.89	2.10 ± 1.62	3.40 ± 2.64	3.3 ± 2.58	4.65 ± 1.55	3.15 ± 2.04	4.45 ± 3.68
Monocytes (% WBC)	3.15 ± 2.46	0.90 ± 0.54 [b]	2.20 ± 1.12	2.10 ± 1.77	2.35 ± 2.66	2.50 ± 1.22	3.80 ± 2.87 [a]	2.20 ± 1.55
Lymphocytes (% WBC)	62.40 ± 8.30	61.70 ± 12.44	60.90 ± 7.17	68.85 ± 6.60	57.50 ± 7.45	59.60 ± 14.80	55.25 ± 5.71	57.55 ± 10.23
Thrombocytes (% WBC)	30.80 ± 7.69	31.30 ± 14.33	34.80 ± 6.33	25.65 ± 6.99	36.85 ± 7.30	33.25 ± 14.74	37.80 ± 7.18	35.80 ± 8.00

Values are expressed as means ± SD (n=10). Different letters mean significant differences among water temperature variations (One-way ANOVA, p ≤0.05)

On triploid fish, several significative cell count variations have been observed, with the most relevant changes being registed on lymphocytes and thrombocytes count, that evolved on a wave form, with lymphocyte percentage hitting their maximum value (72.80 % ± 8.48 %) with a water temperature value of 6.6 ºC, and thrombocytes obtaining highest cell values (47.45 % ± 9.47 %) at 8 ºC. (Table III).

Table III- Relative proportion of triploid *O. mykiss* peripheral blood leukocytes (thrombocytes, lymphocytes, monocytes and neutrophils) for each time sampling

	22.0 ªC	22.0 ºC	15.4 ºC	11.0 ºC	9.2 ºC	6.6 ªC	8.0 ªC	8.0 ªC
Neutrophils (% WBC)	2.85 ± 2.34 [b]	3.35 ± 3.15	3.5 ± 3.49	6.35 ± 3.20 [a]	2.95 ± 1.42	4.85 ± 2.98	3.15 ± 1.69	2.35 ± 0.67
Monocytes (% WBC)	3.15 ± 2.25	1.35 ± 1.07	2.60 ± 1.37	1.85 ± 0.98	2.55 ± 1.82	3.30 ± 1.85	2.25 ± 1.57	2.15 ± 1.21
Lymphocytes (% WBC)	52.95 ± 11.08 [bc]	58.80 ± 6.73[abc]	49.00 ± 9.21[c]	65.75 ± 8.19 [ab]	49.00 ± 10.13 [c]	72.80 ± 8.48 [a]	48.90 ± 9.65[c]	48.05 ± 9.3[c]
Thrombocytes (% WBC)	41.05 ± 9.09 [a]	36.50 ± 7.57[ab]	44.90 ± 9.89[a]	26.05 ± 9.68 [bc]	45.50 ± 11.78 [a]	19.05 ± 7.68 [c]	45.70 ± 9.21[a]	47.45 ± 9.47[a]

Values are expressed as means ± SD (n=10). Different letters mean significant differences among water temperature variations (One-way ANOVA, p ≤0.05).

The medium fish group alterations were observed on neutrophils that have shown higher values at the first two smear group and lymphocyte and thrombocyte population that have produced higher cell proportion on the last sampling times (Table IV).

Table IV- Relative proportion of medium *O. mykiss* peripheral blood leukocytes (thrombocytes, lymphocytes, monocytes and neutrophils) for each time sampling

	22.0 ªC	22.0 ªC	15.4 ºC	11.0 ªC	9.2 ªC	6.6 ªC	8.0 ªC	8.0 ªC
Neutrophils (% WBC)	7.30 ± 4.35 [ab]	7.60 ± 2.85 [a]	1.20 ± 0.87 [c]	4.60 ± 4.64	3.80 ± 2.16	4.60 ± 2.99	1.90 ± 0.80 [bc]	5.20 ± 5.27
Monocytes (% WBC)	3.20 ± 1.50	1.80 ± 0.68	4.30 ± 1.29	1.70 ± 0.68	2.30 ± 1.33	3.20 ± 1.54	3.00 ± 1.76	2.30 ± 0.93
Lymphocytes (% WBC)	56.60 ± 6.30	58.30 ± 4.26	60.80 ± 7.49	53.30 ± 2.40	47.30 ± 6.50 [b]	69.40 ± 10.12 [a]	49.00 ± 6.20 [b]	58.60 ± 8.41
Thrombocytes (% WBC)	32.90 ± 7.50	32.30 ± 3.20	33.70 ± 6.80	40.40 ± 3.84 [a]	46.60 ± 5.34 [a]	22.80 ± 6.42 [b]	46.10 ± 5.40 [a]	33.90 ± 7.70

Values are expressed as means ± SD (n=5). Different letters mean significant differences among water temperature variations (One-way ANOVA, p ≤0.05).

The antiprotease activity has produced significative alterations along temperature variations showing tendency to inhibit more trypsin with cooler water temperatures. Medium sized fish produced no significative differences between time samplings on this parameter (figure 9).

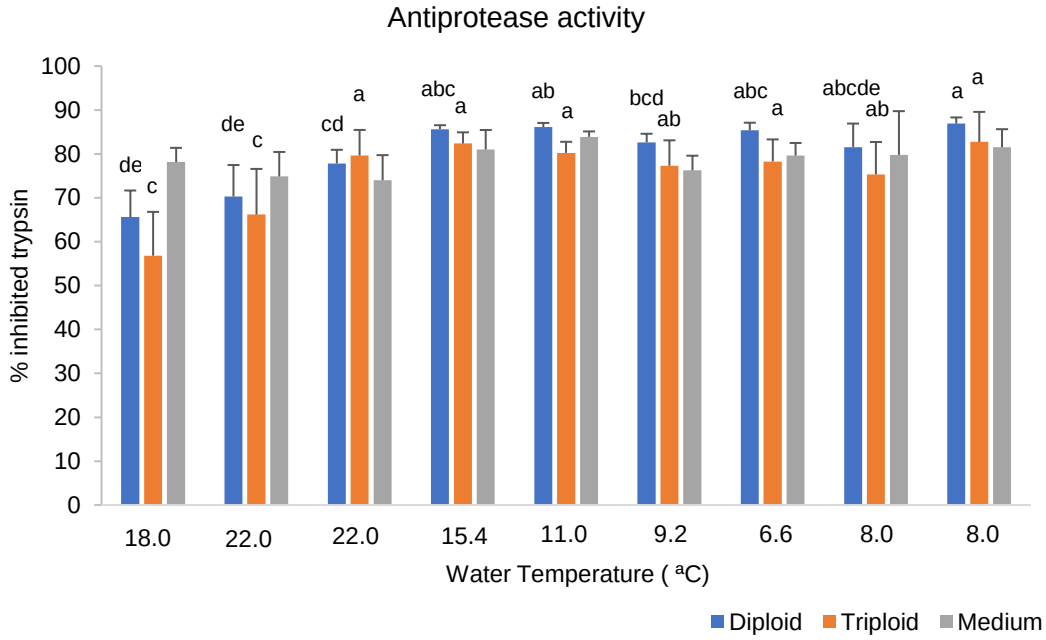

Figure 9- Antiprotease activity of different *O. mykiss* groups along sampling time
Values are expressed as means ± SD (n=10 for diploid and triploid fish and n=5 on medium fish). Different letters mean significant differences among water temperature variations to each group (One-way ANOVA and Kruskal Wallis p ≤0.05).

Lysozyme followed the tendency to increase its concentration with water temperature decrease (figure 10). Highest activity results have been constant to all different groups but different temperatures were observed on the lowest enzyme concentration among the three fish groups.

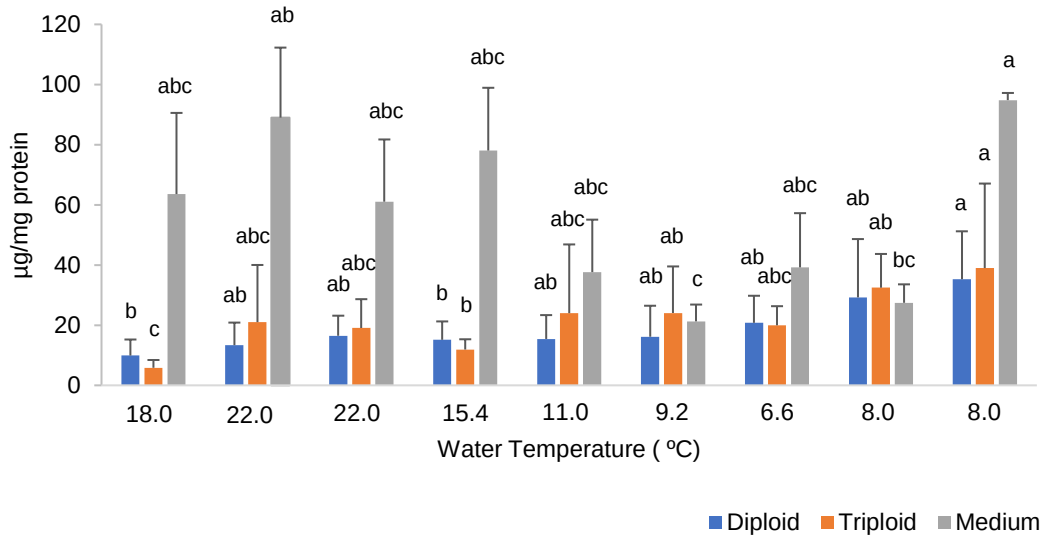

Figure 10- Lysozyme activity of different *O. mykiss* groups along temperature variation.
Values are expressed as means ± SD (n=10 for diploid and triploid fish and n=5 on medium fish). Different letters mean significant differences among water temperature variations to each group (One-way ANOVA and Kruskal Wallis p ≤0.05).

Peroxidase activity has produced opposite results comparing to the other two humoral analysis (figure 11), resulting in highest concentration on warmer temperatures.

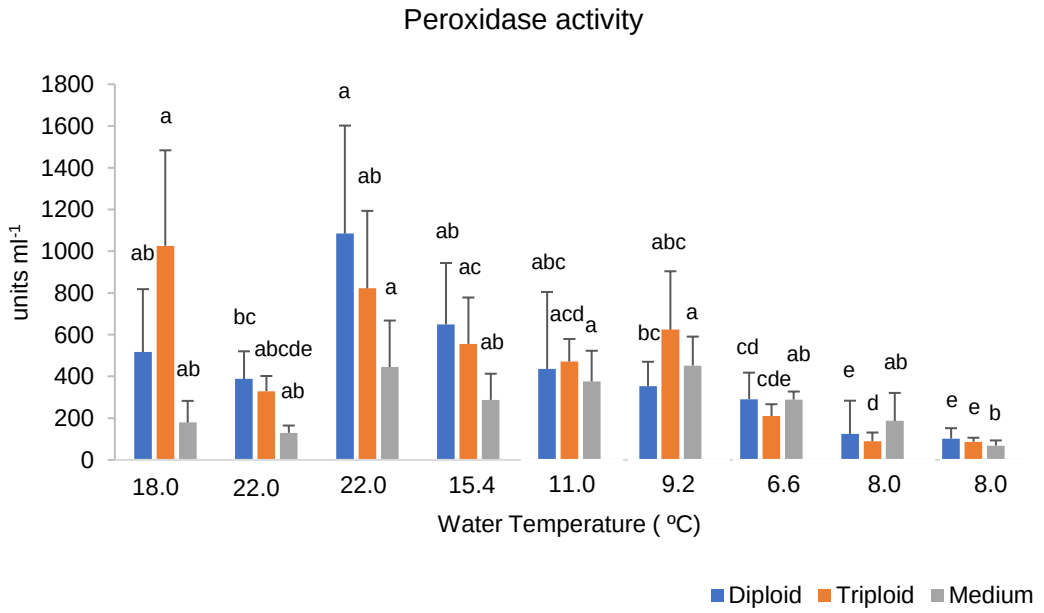

Figure 11 - Peroxidase activity of different *O. mykiss* groups along sampling time.
Values are expressed as means ± SD (n=10 for diploid and triploid fish and n=5 on medium fish). Different letters mean significant differences among water temperature variations to each group(One-way ANOVA and Kruskal Wallis p ≤0.05).

4. Discussion

Haematological results have shown that, on diploid fish, temperature alteration had few influence on blood cell count. However, on triploids, several significative cell count changes have been observed, following the tendency for cell count fluctuation described from past studies on *O. mykiss* (Ojolick *et al.* 1995). On triploid group, neutrophils have significally increased between time 2 and 5, but the major cell type count differences were observed on lymphocytes, that varied on a wave form during time, tending to show highest cell percentage at lower values of water temperature. Thrombocyte counts has suffered variations too, with more relevance for their decrease on September and January. Analogous results on lymphocytes have been observed on other studies with study species (Houston *et al.* 1996), reinforcing the idea that cooler temperatures influentiate positively the number of circulating lymphocyte cells. Already at the medium sized fishes, neutrophils have been highly identified at first and second smear groups, which makes believe that, on this fish group, these cells are more common on summer months. On the opposite sense have evolved thrombocyte cells, whose results registered highest values at lower temperatures, showing similarity to thrombocytary variations on *S. trutta* studies (Rahkonem & Pasternack 1998) and appearing to be the leukocitary cell group with more production rate in cooler waters. After this comparative study, we can consider that water temperature variation affects fish haematological values, specially on triploid and medium fishes, showing highest values of lymphocyte and thrombocyte cells at cooler temperatures, and phagocytic cells being more prevalent on warmer waters.

Already on humoral parameters, antiproteases analysis have showed an increased activity at water temperatures ranges close to optimum rearing temperatures (8-15 ºC), similar to observed data in *D. labrax* that have produced higher antiprotease activity at temperatures between 14 and 19 ºC. (Valero *et al.* 2014). Likewise results observed with environmental temperature variations in *Gadus morhua* (cod) (Magnadóttir et al. 1999), antiprotease activity on *O. mykiss* have also showed relevant decreased values on summer months. Is also relevant to highlight intense activity of this humoral parameter was observed in October, November and March, with more than 85.5 percent of inhibited trypsin being registed. Another curious fact is that diploid fish have produced higher antiprotease mean values than triploid, going along with the results of previous studies on *S. salar* that also produced diploids higher antiprotease concentration than triploids (Langston *et al.* 1997), suggesting that genetic variations might produce different antiprotease activity. It's also remarkable that the group of medium fishes has showed no significant differences during time and presented lower antiprotease concentration than diploid fish, appearing to be the group less affected by our dependent factor, which might suggest that fish age can regulate this enzyme production.

Although it is usually assumed that adaptive immune parameters tend to be suppressed at low temperatures, some studies in *S. aurata* have been reported resulting in increased cellular and humoral immune parameters at cooler temperatures (Machado *et al.* 2015; Guerreiro *et al.* 2016). In our study, fishes have verified their maximum lysozyme concentrations on March, at 8 ºC, with diploid and triploid groups obtaining respectively 35.38 and 38.98 µg per milligram of protein. On a simplistic form, our results provide the idea that, at cooler temperatures (near 10 ºC), phagocytic cells produce more lysozyme, making fish immune system more able to block and lyse bacterial cells, through a modulation still not well understood, but that could be caused by interaction of environmental variations, stress and higher infection incidence. Since monocytes are thought to be the main producers of lysozyme, slight correlation was observed between enzyme concentration and monocyte cell count. Higher monocitary presence and lysozyme reading peaks have been observed on same time samplings, as well as increased average percentage of monocytes on medium group during time, matching with the markable increased enzyme presence on the same sampling group. Considering medium fish group, the evolution of lysozyme concentration has resulted in some contrasts with the diploid and triploid variations along the year, once medium sized fish enzyme values developed on unequal way, with the lowest concentration of lysozyme at 9.2 ºC, on December, and the highest enzyme concentration at 8 ºC, on March, which suggests that this parameter might have been affected not only by water temperature but also from fish growth and maturation. Another recent study on *O. mykiss* has resulted in increased lysozyme concentration along the year occurred due not only by temperature but also by photoperiod (Papežíková *et al.* 2016), which might be another plausible explanation for our results.

Contrarily to the other humoral analysis performed on this thesis, peroxidase concentration has produced the highest concentration values at warmer temperatures, contrasting with lower enzyme scores on cooler water, results that had yet been registered in the past on *Labeo rohita* (roho labeo) species (Swain *et al.* 2007). In our study, little changes were noticed on peroxidase absorbance peaks among groups, with highest enzyme values being obtained on summer months for diploid and triploid fish groups while medium fishes showed strong enzyme activity on summer and autumn sampling months. Although this fact and once lower peroxidase activity is registed on winter months with temperatures near 10 ºC, water temperature seems to clearly modulate this enzyme. Concordance have also been found between myeloperoxidase activity and neutrophils presence on blood smears, with high cell percentage on the same sampling time of high plasmatic presence of the enzyme. Little discordances between these two parameters might occur due to neutrophil temperature dependent activation that leads to degranulation and consequent high presence of myeloperoxidase, without proportional increased cell count. Another explanation could be the increased abundance of

opportunistic bacterial pathogens that might occur due to temperature augmentation, which in turn could induce peroxidase release by activated neutrophils. In order to confirm this hypothesis, a screening of opportunistic pathogens would be necessary.

5. Conclusions

Compilating all data is possible to hypothesize that water temperature has an important role and affects the dynamics of fish cellularity and humoral parameters. As poikilothermic animals, fish's physiological processes are proven to be influenced and modulated by thermic variations and many studies have showed that usually cooler temperatures depress non-specific, humoral and cell-mediated defence mechanisms. The present study was important to show that, on *O. mykiss*, water temperature differentially conditionate leukocitary population and plasmatic immune defences, and that genetic and size differences on animals produce several variations and, occasionally, opposite results. However, it's not well clear how immune defences act isolated and it is important to eliminate other possible factors that, on this study, might have interacted with temperature like pH, water oxygenation, photoperiod, fish growth and weight, fish maturation, nutrition and stress factors.

It was also of relevant interest, to discover if water temperature oscillations can differentially influence healthy and infected animal's immune system, by creation and evaluation of groups in different immunitary condition, once pathogens by their own are proven to immunostimulate fish and few studies have been performed with these characteristics. Further studies can also be performed on the sequence of this project, adding data by analytical procedures to other humoral parameters of plasma such as alternative complement pathway activity, bactericidal activity, nitric oxide, proteases and Immunoglobulin M studies, as well as all mucus analyses which might elucidate and provide stronger results.

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7. Appendix

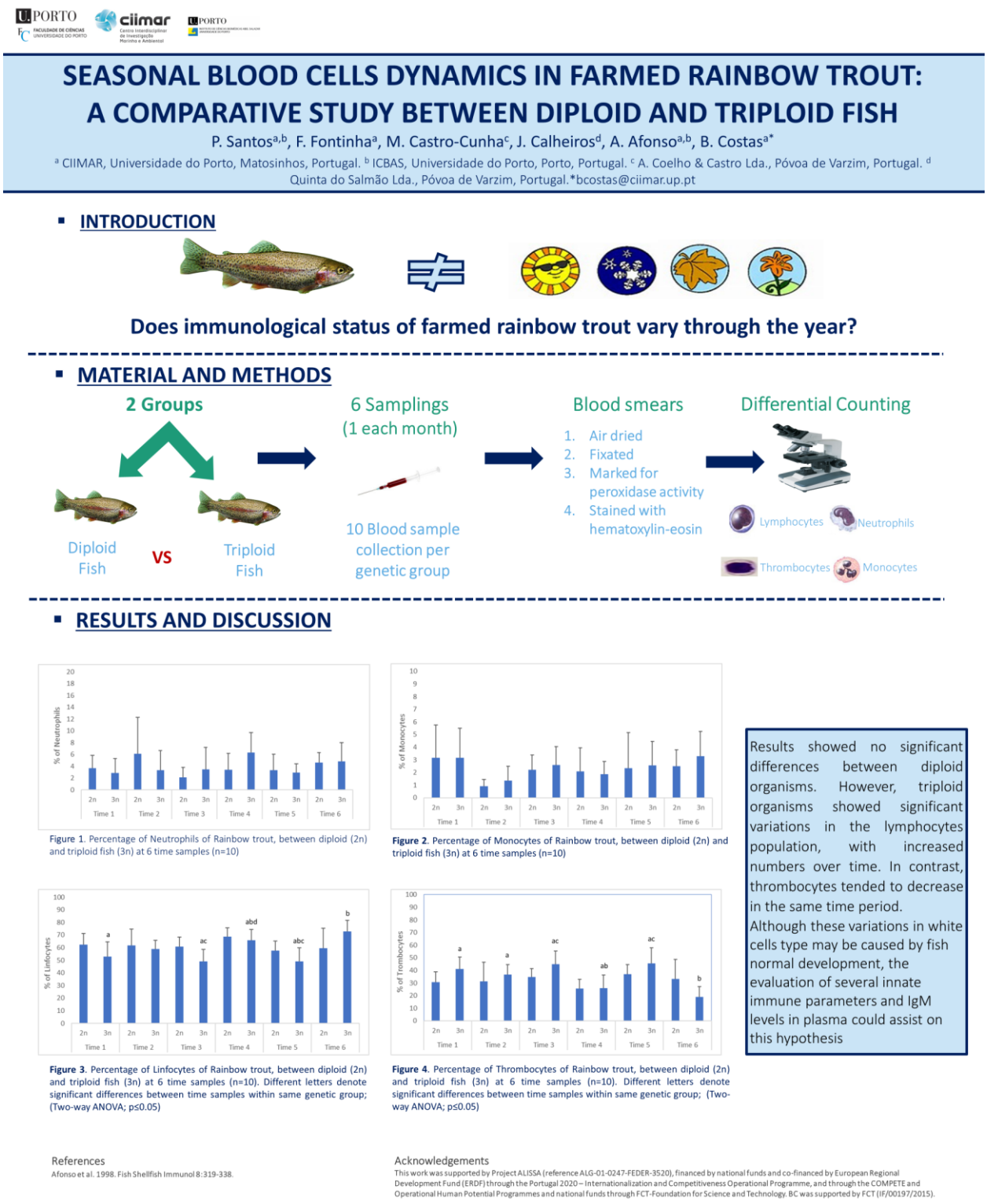

Figure 12- Scientific poster presented at the 2nd Aquaimprove congress, that took place on 17th March, at CIIMAR/UP, Porto, Portugal